Defence Research and Development Canada
Recherche et développement pour la défense Canada

DEFENCE R&D DÉFENSE

Assessment of a Solid Phase Matrix for the Neutralization and Real-Time PCR Detection of *Bacillus anthracis*

D.E. Bader, G.R. Fisher and C.W. Stratilo
DRDC Suffield

Technical Memorandum
DRDC Suffield TM 2006-200
December 2006

Canada

Assessment of a Solid Phase Matrix for the Neutralization and Real-Time PCR Detection of *Bacillus anthracis*

D.E. Bader, G.R. Fisher, and C.W. Stratilo
Defence R&D Canada – Suffield

Defence R&D Canada – Suffield

Technical Memorandum

DRDC Suffield TM 2006-200

December 2006

Author

D.E. Bader

Approved by

Les Nagata

Head, Chemical & Biological Defence Section

Approved for release by

Paul D'Agostino

Chair, Document Review Panel

Abstract

A commercially available, solid-phase DNA binding matrix (FTA® cards) was evaluated for its ability to neutralize live *Bacillus anthracis* and entrap nucleic acid for genetic analysis using real-time polymerase chain reaction (PCR) assays. Cell culture analysis of FTA® cards seeded with live *B. anthracis* indicated that FTA® cards neutralized live *B. anthracis* but at low concentrations. Therefore, FTA® cards spotted with samples containing, or suspected of containing live *B. anthracis* should be considered potentially infectious. PCR analysis of FTA® cards seeded with live *B. anthracis* using assays designed to detect *B. anthracis* plasmidic gene targets, resulted in detection below the live agent "neutralization" concentration. This may be due in part to a number of factors including multiple plasmids present per colony forming unit (cfu), multiple cells per cfu (cellular clumping), and/or, additional gene target contributions from non-viable cells. PCR reaction solutions exposed to discs seeded with low concentrations of live *B. anthracis* were found to be culture-negative and thus may be safe to handle under non-containment conditions, but additional studies would be required to determine the level of safety at higher concentrations. Although FTA® cards exhibited limited neutralization capacity for live *B. anthracis*, they still may be of value for field and lab-based applications as BT/BW agent sample processing, archiving, transport and analysis media, due to their ability to protect and preserve genetic material under storage or transport conditions where cryopreservation (refrigeration or freezing) may not be possible and, therefore, are of continued interest.

Résumé

Une matrice de fixation d'ADN en phase solide (FTA® cards), disponible sur le marché, a été évaluée pour sa capacité à neutraliser le *Bacillus anthracis* vivant et à trapper l'acide nucléide et effectuer une analyse génétique au moyen de bio-essais de réaction en chaîne de la polymérase (PCR) en temps réel. L'analyse de culture de cellules des plaques FTA® semées de *B. anthracis* vivant indique que les plaques FTA® neutralisaient les *B. anthracis* vivants mais à des concentrations faibles. C'est pourquoi on doit estimer que les plaques FTA® maculées d'échantillons contenant ou suspects de contenir du *B. anthracis* vivant sont potentiellement infectieuses. Les analyses PCR des plaques FTA® semées de *B. anthracis* vivants, utilisant des bio-essais conçus pour détecter des cibles de gènes plasmidiques *B. anthracis*, peuvent détecter en dessous de la concentration de « neutralisation » d'agents vivants. Ceci est dû en partie à un certain nombre de facteurs dont des plasmides multiples présents par unité formatrice de colonies (cfu), des cellules multiples par cfu (agglutination cellulaire) et / ou des apports additionnels de cibles géniques provenant de cellules non viables. On a trouvé que les solutions de réaction PCR exposées aux disques semés de *B. anthracis* en faible concentration étaient de culture négative et qu'on pouvait par conséquent les manipuler dans des conditions de non confinement mais que des études supplémentaires seraient requises pour déterminer le niveau sécuritaire à des concentrations plus élevées. Les plaques FTA® démontrent une capacité limitée de neutralisation des *B. anthracis* vivants mais elles continuent toutefois à nous intéresser; elles peuvent être utiles pour les applications, basées en champs ou en laboratoire, de traitement d'échantillons d'agents BT/BW, d'archivage, de transport et d'analyse de milieux; ceci dû à leur capacité de protéger et de préserver les matériaux génétiques stockés ou d'être utilisés dans les conditions de transport où la cryoconservation (réfrigération et congélation) n'est pas possible.

Executive summary

Introduction: A commercially available, solid-phase medium (FTA® cards) has been developed, which, according to its manufacturer (Whatman), is able to capture and retain genetic material from biological samples. It offers the capability to rapidly inactivate many different microbial agents (viruses, bacteria) while protecting genetic material for subsequent analysis over long periods of time (years) at room temperature. This medium is worth investigating for sample processing, archiving, transporting, and analyzing samples that may contain BT/BW agents for both laboratory and field-based applications, particularly where cryopreservation (refrigeration/freezing) is not possible. In this study, FTA® cards were spiked with live *Bacillus anthracis* (Ames strain) and evaluated for their neutralization ability, based on cell culture analysis, and were also analyzed using real-time PCR detection assays designed to detect *B. anthracis* plasmidic gene targets, in order to assess *B. anthracis* detection sensitivity.

Results: No growth was observed for FTA® cards seeded with *B. anthracis* at low concentrations but growth was observed at higher concentrations. Therefore, FTA® cards spotted with samples containing, or suspected of containing, live *B. anthracis* should be considered potentially infectious. PCR analysis of FTA® cards seeded with live *B. anthracis* resulted in detection below the live agent "neutralization" concentration which may be due in part to a number of factors including the presence of multiple plasmids per colony forming unit (cfu), multiple cells per cfu (cellular clumping), and/or, additional gene target contributions from non-viable cells. PCR reaction solutions, exposed to discs seeded with low concentrations of live *B. anthracis*, were found to be culture-negative, and thus may be safe to handle under non-containment conditions, but additional studies would be required to determine the level of safety at higher concentrations.

Significance: Although FTA® cards exhibited limited neutralization capacity for live *B. anthracis*, they still may be of value for field and lab-based applications as BT/BW agent sample processing, archiving, transport, and analysis media, due to their ability to protect and preserve genetic material under storage or transport conditions where cryopreservation (i.e. refrigerated or frozen storage) may not be possible, and therefore, are of continued interest.

Future plans: Experiments are planned to evaluate FTA® cards in terms of sample preservation over time and at various temperatures using PCR.

Bader D.E., Fisher G.R., and Stratilo, C.W. 2006. Assessment of a Solid Phase Matrix for the Neutralization and Real-Time PCR Detection of *Bacillus anthracis*. DRDC Suffield TM 2006-200. Defence R&D Canada – Suffield.

Sommaire

Introduction: Un milieu de culture en phase solide (FTA® cards), disponible sur le marché, a été développé, et est capable selon son manufacturier (Whatman), de capturer et de retenir le matériau génétique à partir d'échantillons biologiques. Il offre la capacité d'inactiver rapidement beaucoup d'agents microbiens différents (virus, bactéries) tout en protégeant le matériau génétique durant de longues périodes (années) à la température ambiante lors des analyses ultérieures. Il serait utile d'étudier ce milieu de culture pour le traitement des échantillons, l'archivage, le transport et l'analyse des échantillons pouvant contenir des agents BT/BW durant les application basées en laboratoire ou en champ, surtout quand la cryoconservation (réfrigération /congélation) n'est pas possible. Dans cette étude, on a semé les plaques FTA® de *Bacillus anthracis* (de souche Ames) vivant. On a évalué leur capacité de neutralisation, en se basant sur l'analyse de la culture de cellule; on les a aussi analysées en utilisant les bio-essais PCR de détection en temps réel, conçus pour détecter les cibles géniques plasmidiques de *B. anthracis*, ceci pour évaluer la sensitivité de la détection de *B. anthracis*.

Résultats: On n'a observé aucune croissance sur les plaques FTA® semées de *B. anthracis* à de faibles concentrations mais on a observé une croissance à de plus hautes concentrations. On doit par conséquent estimer que les plaques FTA®, semées d'échantillons contenant ou suspects de contenir du *B. anthracis* vivant, sont potentiellement infectieuses. Des analyses PCR des plaques FTA® semées de *B. anthracis* vivant ont résulté en une détection en-dessous de la concentration de « neutralisation » d'agent vivant ce qui est dû en partie à un certain nombre de facteurs dont la présence de plasmides multiples per unité de formation de colonies (cfu), de cellules multiples par cfu (agglutination cellulaire) et / ou des apports additionnels de cibles géniques provenant de cellules non viables. On a trouvé que les solutions de réaction PCR exposées aux disques semés de *B. anthracis* en faible concentration étaient de culture négative et qu'on pouvait par conséquent les manipuler dans des conditions de non confinement mais que des études supplémentaires seraient requises pour déterminer le niveau sécuritaire à des concentrations plus élevées.

Portée des résultats: Les plaques FTA® démontrent une capacité limitée de neutralisation des *B. anthracis* vivants mais elles continuent toutefois à nous intéresser; elles peuvent être utiles pour les applications, basées en champs ou en laboratoire, de traitement d'échantillons d'agents BT/BW, d'archivage, de transport et d'analyse de milieux, ceci dû à leur capacité de protéger et de préserver les matériaux génétiques stockés ou d'être utilisés dans les conditions de transport où la cryoconservation (réfrigération et congélation) n'est pas possible.

Plans futurs: On prévoit d'effectuer des expériences avec la méthode PCR pour évaluer les plaques FTA® en termes de préservation d'échantillons, durant une période prolongée et à des températures variées.

Bader D.E., Fisher G.R., and Stratilo, C.W. 2006. Assessment of a Solid Phase Matrix for the Neutralization and Real-Time PCR Detection of *Bacillus anthracis*. DRDC Suffield TM 2006-200. R & D pour la défense Canada – Suffield.

Table of contents

List of tables

List of figures

Introduction

Rapid detection and identification of infectious disease agents that could be used for biowarfare (BW) or bioterrorism (BT), is essential for timely assessment of the threat and the establishment of effective countermeasures. Polymerase chain reaction (PCR) is a basic molecular genetic technique that is used for the detection and identification of such agents [1-7]. PCR is used to amplify genetic sequences of interest and thus requires nucleic acid as a template for the reaction. Intact infectious bioagents must be lysed to release the genetic material (DNA or RNA). Optimal PCR results are obtained when the genetic material is free of PCR inhibitory components and free from degradation (e.g. nuclease digestion, oxidation, UV irradiation etc.). This is accomplished by using various biochemical and physicochemical purification techniques and is often performed in solution. Purified material is then stored in solution at refrigerated temperatures for short term storage (days) or frozen for long term storage (weeks to years) to minimize degradation. Preparation and storage of nucleic acid can be difficult to perform in the field where access to proper equipment and infrastructure may be limited.

A commercially available, solid-phase medium (FTA® cards) has been developed, which according to its manufacturer (Whatman), is chemically treated to lyse cell membranes, entrap nucleic acid, and protect it from nucleases, oxidation and UV damage. It offers the capability to rapidly inactivate many different microbial agents (viruses, bacteria) while protecting genetic material for subsequent analysis over long periods of time (years) at room temperature. This medium is worth investigating for sample processing, archiving, transporting, and analyzing samples that may contain BT/BW agents for both laboratory and field-based applications, particularly where cryopreservation (refrigeration or freezing) is not possible.

Solid-phase, DNA-binding media based on the FTA® card technology have been used for PCR analysis of infectious agents in different biological sample matrices including blood samples [8] and wildlife samples [9]. A comparison study between IsoCode® STIX and FTA® Gene Guard Collection cards for whole blood storage and PCR diagnosis of malaria, demonstrated that both media gave similar results for single-species malaria, but FTA® cards were more sensitive than IsoCode® STIX in mixed infections [8]. At the time of the study, IsoCode® STIX media was marketed by Schleicher & Schuell (S&S) while FTA® Gene Guard Collection cards were marketed by Whatman. In 2003, Whatman initiated steps to stop S&S from exceeding the terms of the licence that had been granted to S&S for the manufacture and sale of its IsoCode® product which was based on Whatman's FTA® technology. In 2004, the two companies settled which resulted in Whatman purchasing the IsoCode® product line and acquiring some patents related to S&S's IsoCode DNA storage products. Presently, only the FTA® product line is commercially available.

Our laboratory recently conducted PCR analysis of FTA® cards seeded with purified total DNA from *Bacillus anthracis* [10] as part of a trinational project between Canada (DRDC Suffield), Sweden (FOI) and the Netherlands (TNO) to investigate this technology for lab and/or field-based applications. While PCR signals from FTA® cards seeded with DNA were observed several cycles later than equivalent amounts of DNA in solution [10], PCR analysis

and neutralization effects of FTA® cards using live agents were considered to be important characteristics for further investigation. Consequently, in this study, FTA® cards were spiked with live *Bacillus anthracis* and evaluated for their neutralization ability based on cell culture analysis and were also analyzed using real-time polymerase chain reaction (real-time PCR) assays to assess *B. anthracis* detection sensitivity. Real-time PCR was performed using assays designed to amplify and detect two *B. anthracis* virulence genes, namely the lethal toxin gene (*lef*) found on plasmid pXO1, and the capsule B gene (*cap*B), located on plasmid pXO2. Real-time PCR assays were conducted using Taqman fluorescent probe detection on a Cepheid Smart Cycler® instrument.

Materials and Methods

Preparation of DNA

Purified *B. anthracis* Ames DNA obtained during a previous study [10] was used in this study as a positive control in real-time PCR assays. A 1 µL volume of Ames DNA at $9.4x10^5$ fg/µL was used per 25 µL PCR reaction (final concentration = $9.4x10^5$ fg/ PCR).

FTA® materials

FTA® Whatman materials used in this study were obtained from VWR International Inc., (Mississauga, ON, CA) or Fisher Scientific Ltd., (Ottawa, ON, CA) (Table 1).

Table 1. FTA® Materials

Item	Catalog Number	Whatman Catalog Number
Indicating FTA® Classic Card	14222-802 (VWR)	WB12-0206
FTA® purification reagent	14222-816 (VWR)	WB12-0204
Harris Micro Punch (2.0 mm) with mat	09-923-354 (Fisher)	WB10-0007
Multi-barrier pouches for classic FTA® cards	14222-834 (VWR)	WB10-0010
Dessicant packets (1 gm)	14222-840 (VWR)	WB10-0003

Preparation and enumeration of live *B. anthracis*

B. anthracis (Ames strain) was aseptically inoculated into 4.0 mL of sterile brain heart infusion (BHI) broth (Difco) in a 15 mL snap cap tube along with a negative control broth tube and allowed to incubate overnight (O/N) in a benchtop shaker incubator at 35°C and 155 rpm (no CO_2) with the snap cap completely closed. One hundred microlitres of O/N culture was aseptically transferred into a fresh sterile 4 mL BHI broth tube and incubated for 4 hr as described above. Ten-fold serial dilutions were prepared from the 4 hr culture (300 µL culture + 2700 µL sterile PBS) from 10^{-1} to 10^{-8}. One hundred µL of each dilution, including the undiluted culture, were inoculated using a micropipet, onto sheep blood agar (SBA) plates that contained tryptic soy agar + 5% sheep blood, pH 7.3 ± 0.2 @ 25°C (PML Microbiologicals, Wilsonville, Oregon, 97070, USA). Each sample was plated in triplicate working from the least concentrated solution to the most concentrated. The plates were incubated at 35°C (no CO2, no shaking) O/N. Colony forming units (cfu) were enumerated the following day.

Spotting FTA® cards with live *B. anthracis*

Indicating FTA® Classic cards were spotted with undiluted and serially diluted *B. anthracis* culture from the same tubes used for the viable cell count. Each card contained four circles that were each spotted in the center of the circle with 100 µL of a given concentration starting with the least concentrated to the most concentrated solution. The cards were allowed to air dry for a minimum of five minutes, then placed into separate Multi-barrier pouches (each containing a one gram dessicant pack) using forceps and then sealed. Pouches were placed into a single Ziploc® freezer bag (17.7 cm x 19.6 cm) which was bleach sprayed (10% bleach) and then placed into a larger Ziploc® freezer bag which was bleach sprayed (10% bleach), ethanol rinsed (80% ETOH), taken outside the biosafety cabinet and stored at room temperature in the BSL3 bacterial suite. Two additional sets of cards were prepared in the same fashion, and stored at 4°C and -70°C (these were not tested in this study). Pipets were treated with 10% bleach and rinsed with 80% ETOH in between each storage temperature card set.

Preparing discs for viability testing and real-time PCR

Viability testing:

Discs were cored from FTA® cards in triplicate starting from the least to most concentrated samples using a 2.0 mm Harris punch. The discs were placed separately into 4 mL of BHI broth and mixed (by hand). The Harris punch was cleaned between each dilution by coring two discs using a clean FTA® card. The broth tubes along with three control tubes (broth only) were placed in a benchtop incubator and incubated with shaking (35°C, 155 rpm, no CO_2, caps closed) for seven days. One hundred microliters from each seven day old broth culture tube were aseptically applied and streaked onto separate SBA plates. The plates were then incubated for an additional seven days (35°C, no shaking, no CO_2).

Real-time PCR:

Nine discs were cored separately for each concentration tested using a 2.0 mm Harris punch (3 for *lef* PCR, 3 for *cap*B PCR and 3 spares). Discs were always prepared starting from the least to most concentrated when more than one concentration was being prepared in the same day in order to minimize the potential for carry over contamination. Each disc was placed into a separate 1.5 mL Simport microfuge tube (VWR Scientific). The Harris punch was cleaned between each dilution by coring two discs using a clean FTA® card. Discs were then washed prior to PCR according to the manufacturer's instructions as follows:

Two hundred µL of FTA® purification reagent was added to each Simport tube and allowed to incubate for a minimum of five minutes at room temperature. The solution was removed by pipet to a waste vial. This step was repeated again for a total of two washes. Two hundred

µL of TE buffer (10 mM Tris-HCl; 0.1 mM EDTA pH 8.0) was added to each Simport tube and allowed to incubate for a minimum of five minutes at room temperature. The solution was removed by pipet to the same waste vial. This step was repeated again for a total of two washes. The Simport tubes were then placed into a dry block (wells filled with water) and heated to 56°C for 10 min and then used directly or stored at room temperature until analyzed by real-time PCR.

Real-time PCR Analysis

Real-time PCR was conducted directly on the washed disc by placing the disc manually into a 25 µL Smart Cycler® tube using a gel loading pipet tip and then adding 25 µL of 1x real-time PCR reaction mixture.

PCR Reaction Mix

The PCR reaction mix was prepared using SmartMix™ HM beads (Cepheid, Sunnyvale, CA, USA). The final reaction component concentrations were 4.2 mM HEPES buffer (pH 7.2), 200 µM dNTPs, 4 mM $MgCl_2$, 3U Hot start Taq Polymerase, 0.25 µM forward primer, 0.25 µM reverse primer, 0.1 µM probe.

Example of a mastermix set up (ten 25 µL rxns @ 1x strength)

SmartMix™ HM beads[a]	5 beads
forward primer (5 µM)	12.5 µL
reverse primer (5 µM)	12.5 µL
probe (2 µM)	12.5 µL
nuclease-free water	212.5 µL

[a] one bead per 50 µL PCR reaction

PCR Primers/Probes

The PCR primers and probes used in this study target the lethal toxin (*lef*) gene on the pXO1 plasmid and the capsule gene (*cap*B) on the pXO2 plasmid of *B. anthracis*.

Lethal toxin gene (*lef*)

CWSLP2 (*lef* forward primer)	5' ggtacaagaagtatttgcgaaagc 3'
CWSRP2b (*lef* reverse primer)	5' atcttgacagcatccgttga 3'
CWSprobe2 (*lef* probe)	5'FAM-tgcatattatatcgagccacagcatcgtga3'BHQ-1
amplicon size	167 bp

Capsule B gene (*cap*B)

CWSRP3 (*cap*B forward primer)	5' gaagcgaaatataagactgtaggg 3'
CWSLP3 (*cap*B reverse primer)	5' ttctaaatcagcagcctctttaac 3'
CWSP3 (*cap*B probe)	5'TET-ctgcgttgctcaccgatattaggaccttc 3'BHQ-1
amplicon size	153 bp

The probes and primers were designed at DRDC Suffield using PREMIER Biosoft Beacon Designer v4.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) and synthesized by Integrated DNA Technologies Inc., Coralville, IA, USA.

Real-time PCR Cycling and Analysis Settings

Real-time PCR was performed on the Smart Cycler® using SCII I-core modules (Cepheid) and ver 2.0d software. The cycling conditions included:

(1) 95°C for 5 min – 1 cycle
(2) 95°C for 15s + 60°C for 60s (with optics "on") – 45 cycles

The data were obtained by the Smart Cycler® software using the analysis settings presented in Table 2 and dye set FTTC25.

Table 2. Smart Cycler® Analysis Settings

Channel	1-FAM; 2-TET
Target	-
Usage	assay
Curve analysis	primary curve
Threshold setting	manual
Manual threshold fluorescence units	30.0
Auto thresh #SD's	N/A
Auto minimum cycle	5
Auto maximum cycle	10
Valid minimum cycle	3
Valid maximum cycle	60
Background subtraction	ON
Boxcar average cycles	0
Background minimum cycles	5
Background maximum cycles	40

Colony Forming Units/PCR Reaction

The number of colony forming units in a PCR reaction (cfu/PCR) was estimated as follows using the highest concentration of agent spotted onto the card as an example. Estimates assume uniform distribution of colony forming units across the entire spot:

(1) # of colony forming units spotted onto the card:

= cfu/mL agent loaded onto the spot x 100 µL (volume of spot)
= 2.4×10^6 cfu/mL x 0.1 mL/spot
= 2.4×10^5 cfu/spot

(2) # colony forming units per PCR:

approx spot diameter (to the edge of the pink zone) = 25 mm
spot radius = 12.5 mm
spot area = 490.6 mm^2

disc diameter = 2 mm
disc radius = 1 mm
disc area = 3.14 mm^2

ratio of disk area to spot area:

$= 3.14\ mm^2 / 490.6\ mm^2$
$= 0.0064$

colony forming units per disk:

$= 2.4 \times 10^5$ cfu/spot x 0.0064
= 1536 cfu/disc
= 1500 cfu/disc (rounded to the nearest 100)

Since the entire disc was used in the PCR reaction the number of cfu/PCR is the same as the number of cfu/disc. These units are used interchangeably in this paper.

Results and Discussion

Viable cell count

The concentration of *B. anthracis* in the original broth culture was found to be $2.4x10^6$ cfu/mL[b] based on the viable cell count obtained by plate culture analysis (Table 3).

Table 3. Viable Cell Count Using Plate Culture Analysis

Sample	# 1	# 2	# 3	Average
PBS	0	0	0	0
BHI broth	0	0	0	0
plate only	0	0	-	0
undiluted	TNTC[c]	TNTC	TNTC	TNTC
10^{-1} dilution	TNTC	TNTC	TNTC	TNTC
10^{-2} dilution	TNTC	TNTC	TNTC	TNTC
10^{-3} dilution	328	228	160	240
10^{-4} dilution	12	14	23	16
10^{-5} dilution	1	2	13	5
10^{-6} dilution	1	0	0	0.33
10^{-7} dilution	0	0	0	0
10^{-8} dilution	0	0	0	0

Viability assessment of FTA® discs seeded with live *B. anthracis*

The results from broth and plate culture analysis of FTA® discs spotted with various concentrations of live *B. anthracis* are presented in Table 4. Visible growth (G) was observed in 2 of 3 replicate broth tubes for the most concentrated sample ($2.4x10^6$ cfu/mL or 1500 cfu/disc) and in 1 of 3 replicate broth tubes for the next most concentrated sample ($2.4x10^5$ cfu/mL or 150 cfu/disc). No growth (NG) was observed for disks seeded with $2.4x10^4$ cfu/mL (15 cfu/disc) or lower. The same results were observed when broth culture was inoculated onto sheep blood agar plates and incubated for seven days. In otherwords, discs that generated growth in broth, generated colonies on plates that were too numerous to count, while discs that generated no growth in broth, resulted in no colonies on the plate.

[b] viable cell count calculation = 240 cfu x 1000 (dilution) / 0.1 mL (inoculum volume)
[c] TNTC = too numerous to count

Table 4. Viability Assessment of FTA® Discs Seeded with Live B. anthracis (Ames)

dilution	cfu/mL[d]	cfu/disc[e]	broth (7 days)			plates (7 days)		
			#1	#2	#3	#1	#2	#3
undiluted	$2.4x10^6$	1500	G[f]	G	NG[g]	TNTC	TNTC	NG
10^{-1}	$2.4x10^5$	150	G	NG	NG	TNTC	NG	NG
10^{-2}	$2.4x10^4$	15	NG	NG	NG	NG	NG	NG
10^{-3}	$2.4x10^3$	1.5	NG	NG	NG	NG	NG	NG
10^{-4}	$2.4x10^2$	0.15	NG	NG	NG	NG	NG	NG
10^{-5}	$2.4x10^1$	0.015	NG	NG	NG	NG	NG	NG
10^{-6}	2.4	0.0015	NG	NG	NG	NG	NG	NG
10^{-7}	0.24	0.00015	NG	NG	NG	NG	NG	NG
10^{-8}	0.024	0.000015	NG	NG	NG	NG	NG	NG

During this study, FTA® card neutralization data were obtained from Whatman product literature [11]. Whatman reported complete inactivation at high titres (10^7 to 10^8 cfu/mL) for bacteria from several different taxa with the exception of *Nocardia*, *Corynebacterium*, *Staphylococcus*, *Bacillus*, *Clostridium*, and *Mycobacterium*. These difficult-to-inactivate bacteria are all Gram-positive bacteria with the exception of *Mycobacteria*, which tend to be Gram-neutral [12], but which have a cell wall that allows them to survive long exposures to acids, alkalis, detergents, and, oxidative bursts [13]. Whatman reported inactivation for representative species from these taxa at lower concentrations ($<10^4$ to 10^5 cfu/mL) which is similar to the neutralization data we obtained for *Bacillus anthracis* (Table 5). In our study, the viability assessment was not performed until at least 48 h after spotting the FTA® cards with live *B. anthracis*, whereas the Whatman data were obtained after only 1 h exposure. Since we observed similar results, it suggests that the neutralization effect is not enhanced by longer exposure times.

Table 5. Comparison of Whatman Neutralization Data to DRDC Suffield Neutralization Data

	Whatman	DRDC Suffield
Neutralization limit	$< 10^4$ to 10^5 cfu/mL	$\leq 2.4x10^4$ cfu/mL
Bacteria	*Nocardia*, *Corynebacterium*, *Staphylococcus*, *Bacillus*, *Clostridium*, *Mycobacterium*	*Bacillus anthracis*
FTA® card exposure	1 hr	48 hr minimum at room temperature
Viability assessment method	streaking disc onto plates	(1) disc into broth (7 days) (2) broth onto plates (7 days)

[d] concentration of *B. anthracis* spotted onto the FTA® cards

[e] based on the area of the 2.0 mm disc (3.14 mm^2) divided by the area of the 100 µL spot (490.6 mm^2) multiplied by the concentration in cfu/mL x 0.1 mL (volume spotted onto the cards)

[f] Growth

[g] No growth

Our data in Table 4 clearly showed that FTA® cards neutralized live *B. anthracis* but only at low concentrations. Other bacterial agents, particularly Gram-negative bacteria and viral agents, may be neutralized at higher concentrations than those observed for *B. anthracis*, however in terms of safety, the default position for FTA® cards spotted with any samples containing, or suspected of containing live BT/BW agents, must be that they should be considered potentially infectious, and thus handled/transported accordingly.

There are ways in which the inactivation of infectious BT/BW agents can be improved prior to spotting the material onto FTA® cards (e.g. heat treatment, chemical treatment, irradiation, etc.). These additional steps, however, add time and complexity to the process, and may create additional issues that may impact their use, particularly for field applications. For example, Whatman has successfully demonstrated inactivation of Gram-positive bacteria at high concentrations (10^8 cfu/mL) using a lysis reagent (L6 buffer) in a five minute pre-treatment step prior to applying the material to the FTA® card. L6 buffer contains the chaotropic agent guanidinium thiocyanate (GuSCN) which has cell lysis and nuclease-inactivating properties [14]. L6 buffer is stable at room temperature in the dark for a minimum of three weeks, but GuSCN will react with acids to produce hydrogen cyanide gas, and therefore, buffers should be prepared in a fumehood. In addition, GuSCN-containing waste should be neutralized with 10N NaOH so that the final concentration does not fall below 0.3N. Thus the L6 buffer, although effective, has a short shelf life, poses a potential hazard when mixed with acids, and generates waste that must be discarded. These limitations may be acceptable in the laboratory but they may not be acceptable in the field.

Of additional interest to us during this study, was the question as to whether one might observe viable cell growth from the contents of the PCR reaction tube following PCR analysis of agent-seeded discs, in the event one may wish to perform subsequent analysis of the amplified material under non-containment conditions. Unfortunately, the discs could not be easily removed from the Smart Cycler® tubes, so only the PCR solution could be tested for the presence of viable cells. Consequently, PCR solution (~ 25 µL) from Smart Cycler® tubes containing discs in which viable cell growth was demonstrated, was transferred to separate SBA plates, streaked, and then incubated for extended periods (7 days for *cap*B PCR reactions, 6 days for *lef* PCR reactions). No colonies were observed on any of the plates, for any of the samples, suggesting that the PCR reaction solution in the PCR reaction tube of discs seeded with low concentrations of live *B. anthracis* may be safe to handle under non-containment conditions. Obviously, additional studies would be required to determine the level of safety at higher concentrations.

Real-time PCR assessment of FTA® discs seeded with live *B. anthracis*

Table 6 summarizes the data obtained from real-time PCR analysis of FTA® discs seeded with various amounts of live *B. anthracis*.

Table 6. Real-time PCR of FTA® 2.0 mm Discs Seeded with Live B. anthracis (Ames)

dilution	cfu/mL	cfu/PCR	culture results	*cap*B Ct[h]	*cap*B replicates	*lef* Ct	*lef* replicates
undil	$2.4x10^6$	1500	positive	19.95 ± 0.32	3/3	20.06 ± 0.47	3/3
10^{-1}	$2.4x10^5$	150	positive	22.50 ± 0.53	3/3	22.85 ± 1.46	3/3
10^{-2}	$2.4x10^4$	15	negative	26.34 ± 1.05	3/3	27.43 ± 0.11	3/3
10^{-3}	$2.4x10^3$	1.5	negative	30.21 ± 0.70	3/3	31.19 ± 1.49	3/3
10^{-4}	$2.4x10^2$	0.15	negative	34.06 ± 1.53	2/6	34.89 ± 4.04	4/6
10^{-5}	$2.4x10^1$	0.015	negative	0.00 ± 0.00	7/7	0.00 ± 0.00	6/6
10^{-6}	2.4	0.0015	negative	0.00 ± 0.00	3/3	0.00 ± 0.00	3/3
PC[i]				24.41 ± 0.60	9/9	25.19 ± 0.44	9/9
ntc[j]				0.00 ± 0.00	9/9	0.00 ± 0.00	9/9

The real-time PCR data provided evidence that there were more copies of the target present in the PCR reaction than was indicated by the viable cell count. First, a PCR signal was observed not only for culture-positive discs, but also for culture-negative discs. The lowest concentration of *B. anthracis* that was PCR-positive and culture-negative, was $2.4x10^2$ cfu/mL or 0.15 cfu/PCR (2 of 6 replicates). For gene targets that are present as a single copy per cfu, it is not theoretically possible to detect less than one cfu/PCR reaction and it can be difficult to detect even 1 cfu/PCR reaction in practice, yet we detected a signal at 0.15 cfu/PCR reaction based on the viable cell count. Second, if one compares the difference between the observed Ct values and the expected Ct values derived from standard curves (Annex A), we observed an average ΔCt value of about 10.6 cycles (10.78 for *cap*B and 10.41 for *lef*) (Table 7).

Table 7. Observed and Calculated Ct Values Based on the Viable Cell Count

			*cap*B Ct values			*lef* Ct values		
dilution	cfu/mL	cfu/disc	observed	calculated[k]	ΔCt	observed	calculated	ΔCt
undil	$2.4x10^6$	1500	19.95	30.44	10.49	20.06	30.67	10.61
10^{-1}	$2.4x10^5$	150	22.50	33.92	11.42	22.85	34.18	11.33
10^{-2}	$2.4x10^4$	15	26.34	37.40	11.06	27.43	37.70	10.27
10^{-3}	$2.4x10^3$	1.5	30.21	40.87	10.66	31.19	41.20	10.01
10^{-4}	$2.4x10^2$	0.15	34.06	44.34	10.28	34.89	44.71	9.82
average					10.78			10.41
PC			24.41	25.31	0.90	25.19	25.48	0.29

[h] Ct values represent the cycle number at which the fluorescence rises above the manual threshold fluorescence unit setpoint which was set at 30 (default setting).

[i] PC = positive control (total DNA from *Bacillus anthracis* Ames strain @ $1x10^5$ copies/PCR reaction)

[j] ntc = no template control

[k] Calculated Ct values were derived from the standard curves (Annex A) based on a total mol wt of $3.63x10^9$ g/mol per cfu multiplied by cfu/disc. This calculation assumes 1 molecule of target DNA per cfu.

This is equivalent to an average of 1500-fold difference ($2^{10.6}$) between the measured Ct value and the expected Ct value based on a doubling of template following every PCR cycle. The data presented in Table 6 and 7 support the notion that that there was more target material in the sample than the viable cell counts indicated. It is interesting to note that the ΔCt values for the positive control DNA for both the *lef* and *cap*B assays, were less than a single cycle, indicating that the Ct values obtained for positive control DNA analyzed on the Smart Cycler in the BSL3 bacterial suite, were very close to the Ct values obtained for the same positive control DNA analyzed on a different Smart Cycler in BSL2 [10].

One possible explanation for better than expected detection sensitivities of FTA® cards seeded with live agent, may be due to the presence of more than one copy of the target gene per cfu. The genes targeted in the *lef* assay and the *cap*B assay are located on the pXO1 and pXO2 plasmids, respectively. These plasmids can be present in multiple copies in the cell. A recent study of 32 *B. anthracis* strains/isolates found between 24 and 243 copies for the pXO1 plasmid (*lef* gene) and between 1 and 32 copies of the pXO2 plasmid (*cap*B gene) [15]. Plasmid copy number cannot be the only factor that contributed to better than expected detection sensitivity. Another contributing factor may have resulted from an underestimation of cells from the viable cell count due to the possibility of multiple cells giving rise to a single cfu as a consequence of cellular clumping. Visible aggregation of cells was observed in the overnight culture, providing evidence of cellular clumping at the macroscopic level, despite extensive vortexing to disperse and solubilize the aggregated cell mass. Even though the "solubilized" portion of the culture was used in this study, cellular clumping could still occur at the microscopic level resulting in multiple cells giving rise to a single viable cfu. In addition to cellular clumping, plasmidic material from non-viable (dead) cells in the culture matrix, as a consequence of normal cell death during bacterial growth and replication, may have contributed additional gene target material that could not be accounted for based on the viable cell count.

Although FTA® cards have limitations with respect to BT/BW agent-neutralization capacity, they still may be of value for field and lab-based applications as BT/BW agent sample processing, archiving, transport, and analysis media, due to their ability to protect and preserve genetic material under storage or transport conditions where cryopreservation (i.e. refrigeration or freezing) may not be possible, and therefore, are of continued interest. GenVault® Corporation (Carlsbad, California) has recently developed a multi-well, DNA sample storage and retrieval management system based on Whatman FTA® isolation and preservation media to meet high throughput DNA storage demands, while eliminating the need for cryopreservation. They have integrated a DNA-based biological sample tracking method that allows one to label samples biologically using a combination of oligonucleotides (referred to as GenCode) that can be co-eluted with the DNA sample in question, thereby eliminating sample misidentification errors [16]. Up to as many as one billion unique codes can be generated for use as permanent sample identifiers. GenVault has also improved the DNA recovery efficiency from FTA® media by up to 70% (Larry Cohen, GenVault Corporation, personal communication) which makes this technology worthy of continued interest.

Conclusion

Cell culture analysis of FTA® cards seeded with live *B. anthracis* indicated that FTA® cards neutralized live *B. anthracis* but at low concentrations. Therefore, FTA® cards spotted with samples containing, or suspected of containing live *B. anthracis* should be considered potentially infectious, and handled accordingly. Real-time PCR analysis of FTA® cards seeded with live *B. anthracis* resulted in detection limits below the live agent "neutralization" concentration which may be due in part to a number of factors including multiple plasmids present per colony forming unit (cfu), multiple cells per cfu (cellular clumping), and/or, additional gene target contributions from non-viable cells. PCR reaction solutions exposed to discs seeded with low concentrations of live *B. anthracis*, were found to be culture-negative and thus may be safe to handle under non-containment conditions, but additional studies would be required to determine the level of safety at higher concentrations. Although FTA® cards exhibited limited neutralization capacity for live *B. anthracis*, they still may be of value for field and lab-based applications as BT/BW agent sample processing, archiving, transport and analysis media, due to their ability to protect and preserve genetic material under storage or transport conditions where cryopreservation (refrigeration or freezing) may not be possible, and therefore, are of continued interest.

References

1. Christensen, D.R., Hartman, LJ., Loveless, B.M., Frye, M.S., Shipley, M.A., Bridge, D.L., Richards, M.J., Kaplan, R.S., Garrison, J., Baldwin, C.D., Kulesh, D.A., and Norwood, D.A. (2006). Detection of Biological Threat Agents by Real-Time PCR: Comparison of Assay Performance on the R.A.P.I.D., the LightCycler, and the Smart Cycler Platforms. Clin. Chemistry, v. 52 pp. 141-145.

2. Mothershed, E.A. and Whitney, A.M. (2006). Nucleic Acid-Based Methods for the Detection of Bacterial Pathogens: Present and Future Considerations for the Clinical Laboratory. Clin. Chim. Acta., v. 363, pp. 206-220.

3. Lim, D.V., Simpson, J.M., Kearns, E.A. and Kramer, M.F.(2005). Current and Developing Technologies for Monitoring Agents of Bioterrorism and Biowarfare. Clin. Micro. Rev. v. 18, pp. 583-607.

4. Dragon, D.C., Bader, D.E., Mitchell, J., and Woollen, N. (2005). Natural Dissemination of *Bacillus anthracis* Spores in Northern Canada. App. Env. Micro., v. 71, pp. 1610-1615.

5. Bader, D.E. and Fisher, G.R. (2004). PCR Assays for BW Agent Analysis using Fluorescence-based SYBR® Green Detection. (DRDC Suffield TM 2004-218). Defence R&D Canada – Suffield.

6. Bader D.E., Fisher G.R., and McLaws, L.J. (2004). PCR Analysis of a Simulated Biological/Chemical Agent Mixture in Soil. (DRDC Suffield TM 2004-164). Defence R&D Canada – Suffield.

7. Ivnitski, D., O'Neil, D.J., Gattuso, A., Schlicht, R., Calidonna, M., Fisher, R. (2003). Nucleic Acid Approaches for Detection and Identification of Biological Warfare and Infectious Disease Agents. Biotechniques, v. 35, pp. 862-869.

8. Zhong, K.Z.Y., Salas C.J., Shafer, R., Gubanov, A., Gasser Jr., R.A., Magill, A.J., Forney, J.R., and Kain, K.C. (2001). Comparison of IsoCode STIX and FTA Gene Guard Collection Matrices as Whole-Blood Storage and Processing Devices for Diagnosis of Malaria by PCR. Journal of Clinical Microbiology, v. 39, pp. 1195-1196.

9. Smith, L.M. and Burgoyne, L.A. (2004). Collecting, Archiving and Processing DNA from Wildlife Samples Using FTA® Databasing Paper. BMC Ecology, v. 4, http://www.biomedcentral.com/1472-6785/4/4.

10. Bader, D.E., Fisher. G.R., and Stratilo, C.W. Evaluation of a Solid Phase DNA Binding Matrix for Downstream PCR Analysis. (DRDC Suffield TM 2005-226). Defence R&D Canada – Suffield.

11. Whatman FTA® for Microbial Inactivation. (Online) Whatman®. http://www.whatman.com/repository/documents/s7/WGP_170_V7.pdf (Dec 2006).

12. Trifiro, S., Bourgault, A-M., Lebel, F., Rene, P. (1990). Ghost Mycobacteria on Gram Stain. Journal of Clinical Microbiology, v. 28, pp. 146-147.

13. Wikipedia contributors. Mycobacterium. Wikepedia, The Free Encyclopedia. http://en.wikipedia.org/w/index.php?title=Mycobacterium&oldid=98514772 (Dec 2006).

14. Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-van Dillen, P.M.E., and van der Noordaa, J. (1990). Rapid and Simple Method for Purification of Nucleic Acids. Journal of Clinical Microbiology, v. 28, pp. 495-503.

15. Coker, P.R., Smith, K.L., Fellows, P.F., Rybachuck, G., Kousoulas, K.G., and Hugh-Jones, M.E. (2003). *Bacillus anthracis* Virulence in Guinea Pigs Vaccinated with Anthrax Vaccine Adsorbed Is Linked to Plasmid Quantities and Clonality. Journal of Clinical Microbiology, v. 41, pp. 1212-1218.

16. Davis, J.C., Shon, J., Wong D.T., Jaffe, S., and McEvoy, J. (2005). A DNA-Based Biological Sample Tracking Method. Cell Preservation Technology, v. 3, pp. 54-60. Mary Ann Liebert, Inc.

List of symbols/abbreviations/acronyms/initialisms

BT	bioterrorism
BW	biological warfare
cfu	colony forming unit
Ct	cycle threshold
DNA	deoxyribonucleic acid
G	growth
NG	no growth
ntc	no template control
PC	positive control
PCR	polymerase chain reaction
RNA	ribonucleic acid
TNTC	too numerous to count

Annex A – Figures

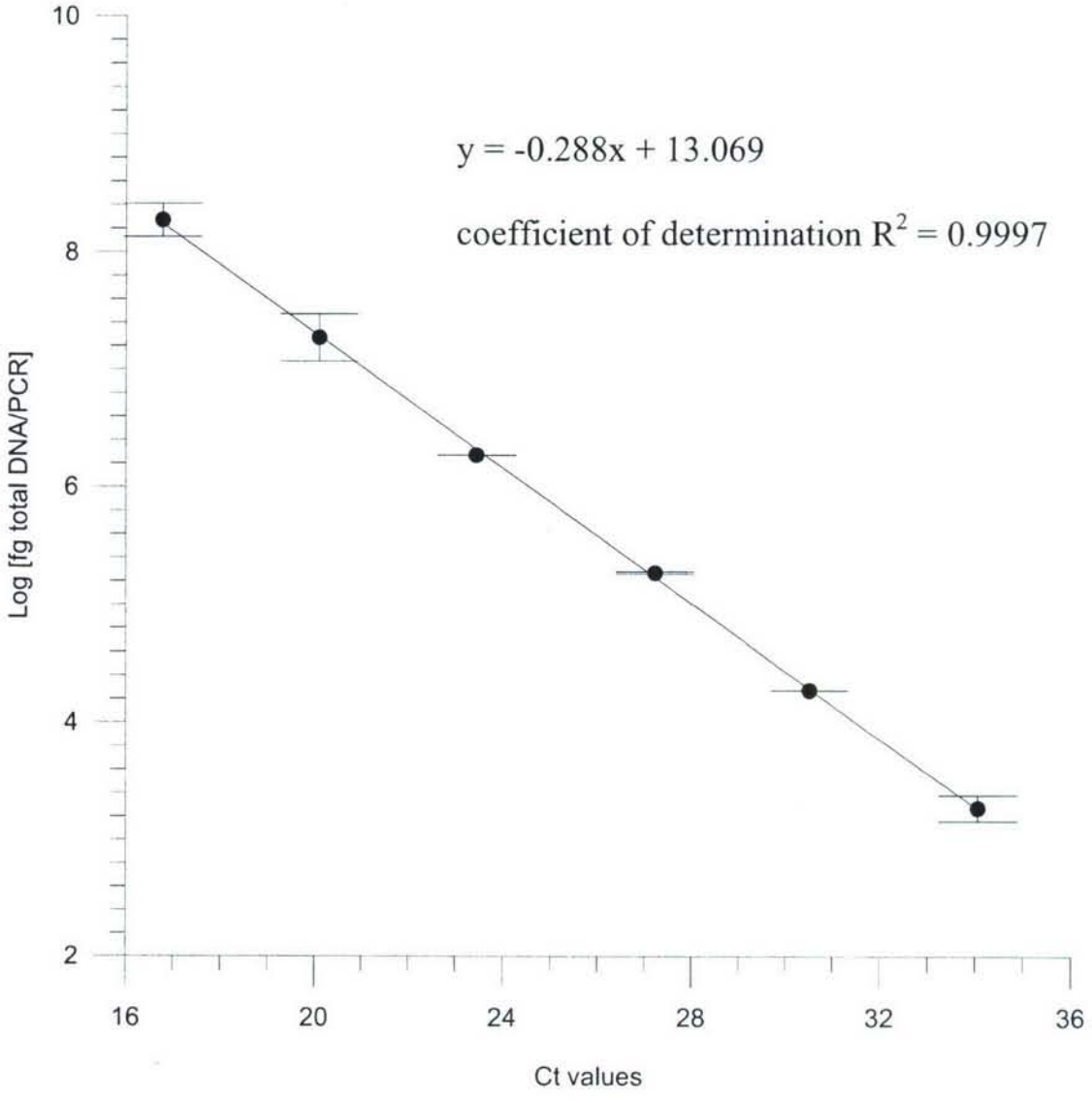

Figure 1. Standard Curve for the *cap*B Assay using *B. anthracis* Ames total DNA in solution

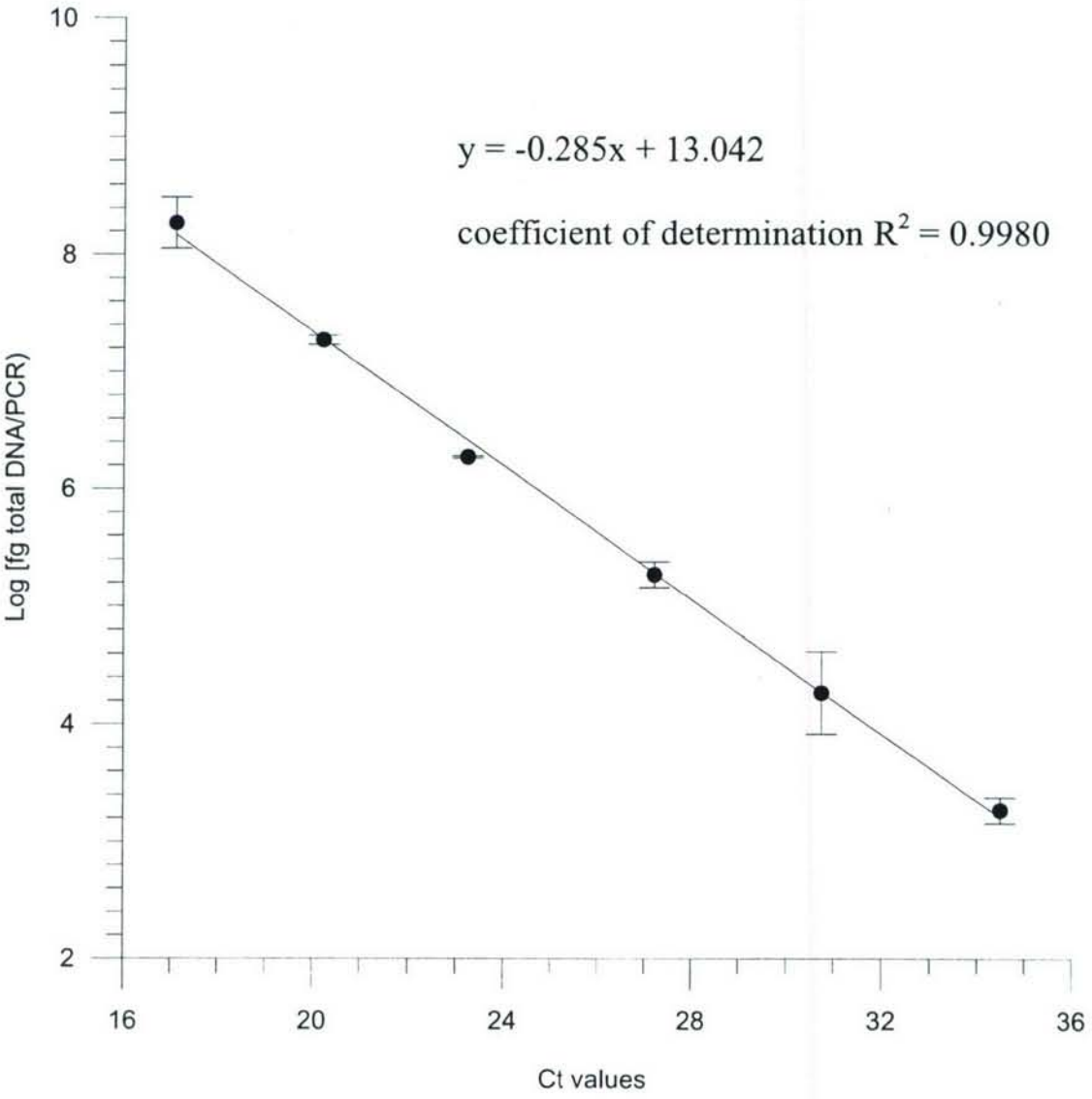

Figure 2. Standard Curve for the *lef* Assay using *B. anthracis* Ames total DNA in solution

DOCUMENT CONTROL DATA

(Security classification of title, body of abstract and indexing annotation must be entered when the overall document is classified)

1. ORIGINATOR (the name and address of the organization preparing the document. Organizations for who the document was prepared, e.g. Establishment sponsoring a contractor's report, or tasking agency, are entered in Section 8.)

   DRDC Suffield, P.O. Box 4000, Stn Main, Medicine Hat, AB, T1A 8K6

2. SECURITY CLASSIFICATION (overall security classification of the document, including special warning terms if applicable)

   UNCLASSIFIED

3. TITLE (the complete document title as indicated on the title page. Its classification should be indicated by the appropriate abbreviation (S, C or U) in parentheses after the title).

   Assessment of a Solid Phase Matrix for the Neutralization and Real-Time PCR Detection of *Bacillus anthracis*. (U)

4. AUTHORS (Last name, first name, middle initial. If military, show rank, e.g. Doe, Maj. John E.)

   Bader, Douglas E., Fisher, Glen R., and Stratilo, Chad W.

5. DATE OF PUBLICATION (month and year of publication of document)

   December 2006

6a. NO. OF PAGES (total containing information, include Annexes, Appendices, etc) 25

6b. NO. OF REFS (total cited in document) 16

7. DESCRIPTIVE NOTES (the category of the document, e.g. technical report, technical note or memorandum. If appropriate, enter the type of report, e.g. interim, progress, summary, annual or final. Give the inclusive dates when a specific reporting period is covered.)

   Technical Memorandum

8. SPONSORING ACTIVITY (the name of the department project office or laboratory sponsoring the research and development. Include the address.)

9a. PROJECT OR GRANT NO. (If appropriate, the applicable research and development project or grant number under which the document was written. Please specify whether project or grant.)

   16QD33

9b. CONTRACT NO. (If appropriate, the applicable number under which the document was written.)

10a. ORIGINATOR'S DOCUMENT NUMBER (the official document number by which the document is identified by the originating activity. This number must be unique to this document.)

   DRDC Suffield TM 2006-200

10b. OTHER DOCUMENT NOs. (Any other numbers which may be assigned this document either by the originator or by the sponsor.)

11. DOCUMENT AVAILABILITY (any limitations on further dissemination of the document, other than those imposed by security classification)

   (X) Unlimited distribution
   ( ) Distribution limited to defence departments and defence contractors; further distribution only as approved
   ( ) Distribution limited to defence departments and Canadian defence contractors; further distribution only as approved
   ( ) Distribution limited to government departments and agencies; further distribution only as approved
   ( ) Distribution limited to defence departments; further distribution only as approved
   ( ) Other (please specify):

12. DOCUMENT ANNOUNCEMENT (any limitation to the bibliographic announcement of this document. This will normally corresponded to the Document Availability (11). However, where further distribution (beyond the audience specified in 11) is possible, a wider announcement audience may be selected).

   Unlimited

13. ABSTRACT (a brief and factual summary of the document. It may also appear elsewhere in the body of the document itself. It is highly desirable that the abstract of classified documents be unclassified. Each paragraph of the abstract shall begin with an indication of the security classification of the information in the paragraph (unless the document itself is unclassified) represented as (S), (C) or (U). It is not necessary to include here abstracts in both official languages unless the text is bilingual).

A commercially available, solid-phase DNA binding matrix (FTA® cards) was evaluated for its ability to neutralize live *Bacillus anthracis* and entrap nucleic acid for genetic analysis using real-time polymerase chain reaction (PCR) assays. Cell culture analysis of FTA® cards seeded with live *B. anthracis* indicated that FTA® cards neutralized live *B. anthracis* but at low concentrations. Therefore, FTA® cards spotted with samples containing, or suspected of containing live *B. anthracis* should be considered potentially infectious. PCR analysis of FTA® cards seeded with live *B. anthracis* using assays designed to detect *B. anthracis* plasmidic gene targets, resulted in detection below the live agent "neutralization" concentration. This may be due in part to a number of factors including multiple plasmids present per colony forming unit (cfu), multiple cells per cfu (cellular clumping), and/or, additional gene target contributions from non-viable cells. PCR reaction solutions exposed to discs seeded with low concentrations of live *B. anthracis* were found to be culture-negative and thus may be safe to handle under non-containment conditions, but additional studies would be required to determine the level of safety at higher concentrations. Although FTA® cards exhibited limited neutralization capacity for live *B. anthracis*, they still may be of value for field and lab-based applications as BT/BW agent sample processing, archiving, transport and analysis media, due to their ability to protect and preserve genetic material under storage or transport conditions where cryopreservation (refrigeration or freezing) may not be possible and, therefore, are of continued interest.

14. KEYWORDS, DESCRIPTORS or IDENTIFIERS (technically meaningful terms or short phrases that characterize a document and could be helpful in cataloguing the document. They should be selected so that no security classification is required. Identifies, such as equipment model designation, trade name, military project code name, geographic location may also be included. If possible keywords should be selected from a published thesaurus, e.g. Thesaurus of Engineering and Scientific Terms (TEST) and that thesaurus-identified. If it is not possible to select indexing terms which are Unclassified, the classification of each should be indicated as with the title.)

Bacillus anthracis, DNA, genetic analysis, real-time polymerase chain reaction, PCR, solid-phase DNA binding matrix

Defence R&D Canada

Canada's Leader in Defence
and National Security
Science and Technology

R & D pour la défense Canada

Chef de file au Canada en matière
de science et de technologie pour
la défense et la sécurité nationale

www.drdc-rddc.gc.ca